



Interleukin-32 upregulates the expression of ABCA1 and ABCG1 resulting in reduced intracellular lipid concentrations in primary human hepatocytes

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ABSTRACT

Background and aims: The role of interleukin (IL-)32 in inflammatory conditions is well-established, however, the mechanism behind its role in atherosclerosis remains unexplained. Our group reported a promoter single nucleotide polymorphism in IL-32 associated with higher high-density lipoprotein (HDL) concentrations. We hypothesize that endogenous IL-32 in liver cells, a human monocytic cell line and carotid plaque tissue, can affect atherosclerosis by regulating (HDL) cholesterol homeostasis via expression of cholesterol transporters/mediators.

Methods: Human primary liver cells were stimulated with recombinant human (rh)TNF α and poly I:C to study the expression of IL-32 and mediators in cholesterol pathways. Additionally, IL-32 was overexpressed in HepG2 cells and overexpressed and silenced in THP-1 cells to study the direct effect of IL-32 on cholesterol transporters expression and function.

Results: Stimulation of human primary liver cells resulted in induction of *IL-32 α* , *IL-32 β* and *IL-32 γ* mRNA expression ($p < 0.01$). A strong correlation between the expression of IL-32 γ and ABCA1, ABCG1, LXR α and apoA1 was observed ($p < 0.01$), and intracellular lipid concentrations were reduced in the presence of endogenous IL-32 ($p < 0.05$). Finally, *IL32 γ* and *ABCA1* mRNA expression was upregulated in carotid plaque tissue and when *IL-32* was silenced in THP-1 cells, mRNA expression of *ABCA1* was strongly reduced.

Conclusions: Regulation of IL-32 in human primary liver cells, HepG2 and THP-1 cells strongly influences the mRNA expression of *ABCA1*, *ABCG1*, *LXR α* and *apoA1* and affects intracellular lipid concentrations in the presence of endogenous IL-32. These data, for the first time, show an important role for *IL32* in cholesterol homeostasis.

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1. Introduction

Cardiovascular diseases (CVD) are currently the leading cause of

death in developed countries, with atherosclerosis as the most important contributor to the disease burden [1,2]. Atherosclerosis is characterized by inflammation and accumulation of lipids in the vessel wall causing plaque formation [3]. Additionally, triggers such as, smoking, hypertension, dyslipidemia and hyperglycemia, are known to contribute to plaque formation [3–5]. Ongoing inflammation in the plaque further activates monocytes to differentiate into macrophages, which will take up lipids to generate foam cells. Moreover, many studies have indicated that pro-inflammatory

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cytokines, including TNF α , IL-1 β , IL-6, IFN γ , contribute to the development of atherosclerosis [6–8]. Recently, Heinhuis et al. suggested that the intracellular pro-inflammatory cytokine interleukin (IL)-32 could play an important role in atherosclerosis [9]. IL-32 has been shown to play a role in inflammatory diseases with an increased risk for CVD, such as rheumatoid arthritis (RA) and human immunodeficiency virus (HIV) [10–13]. IL-32 can be spliced into various isoforms with IL-32 α , IL-32 β and IL-32 γ being most intensively studied and IL-32 γ being the most active isoform [14]. In atherosclerotic plaques, IL32 is expressed, and in macrophages, IL32 overexpression increases the expression of chemokine (C-C motif) ligand 2 (CCL2), soluble vascular cell adhesion molecule (sVCAM-1), matrix metalloproteinase 1 (MMP1), MMP9, and MMP13. IL-32 promotes inflammation by induction of pro-inflammatory cytokines like TNF α , IL-6, IL-1 β and IL-8 [15,16]. Furthermore, IL-32 seems to be a regulator of endothelial cell function where it enhances IL-1 β -induced intracellular adhesion molecule 1 (ICAM-1) [17].

In contrast to these pro-atherogenic actions, IL32 could also have anti-atherosclerotic effects by increased HDL cholesterol [18]. Recently, a promoter single nucleotide polymorphism (SNP) in *IL32* was found to be associated with HDL cholesterol (HDLc) concentrations in both RA patients as well as individuals with an increased CVD risk, again suggesting a role for IL-32 in CVD [18]. Individuals homozygous for the C- allele showed higher HDLc concentrations compared to individuals being heterozygous or homozygous for the T-allele. HDLc is considered to be atheroprotective even though Mendelian randomization studies and drug trials showed there is no causality dependent on HDL cholesterol levels but rather atheroprotective HDL functions are more relevant metrics to analyze [19,20]. Therefore, *IL32* could still have an atheroprotective role. The HDL metabolism starts in the liver, which is an important organ in HDL biosynthesis and a regulator of plasma HDL concentrations. Additionally, the small intestine, and especially enterocytes are involved in biosynthesis of HDLc. Dietary lipids including cholesterol esters and triacylglycerols are first hydrolyzed in the interstitial lumen of the intestine after which the products are taken up by enterocytes [21]. These products are re-synthesized by enterocytes and packed into either chylomicrons or HDLc for secretion into the circulation. Moreover, the biosynthesis of HDL involves synthesis and secretion of apolipoproteins (apoA-I and apoA-II) followed by acquisition of lipids and generation of mature HDL [22,23]. Lipidation of nascent, discoidal apoA-I containing HDL particles must occur to form mature HDL. A critical participant herein is the ATP-binding cassette A1 (ABCA1) expressed on liver cells and enterocytes. These nascent, discoidal apoA-I containing HDL particles are secreted via hepatic and enterocyte ABCA1 and are matured in circulation via the lecithine-cholesterol-acyltransferase (LCAT) function and formation of spherical HDL particles [24]. In the absence of ABCA1, extremely low levels of HDLc and apoA-I are observed, which contribute to an increased risk for CVD.

An important mechanism involved in regulation of excessive cholesterol is reverse cholesterol transport (RCT). During RCT, excessive cholesterol is transferred from peripheral tissues and the arterial wall back to the liver for removal from the body [25,26]. This cholesterol efflux is also mediated via ABCA1, which is induced by activation of liver X receptor alpha (LXR α) [27–29]. Alternatively, ABCG1 transports excessive cholesterol outside the cells [30,31]. Upon return to the liver, HDLc can be taken up by scavenger receptor class B, type 1 (SR-B1) for degradation of HDL. Another alternative pathway, by which HDLc is metabolized and transported to the liver, is via the cholesteryl ester (CE) transfer protein (CETP). Knowing the effect of IL-32 on HDLc concentrations and the importance of the role of the liver in HDLc and cholesterol efflux,

one can argue a role for IL-32 in HDLc metabolism and synthesis in liver cells. However, despite previous studies on the role of IL-32 in cardiovascular disease, studies investigating the exact mechanism of how IL-32 influences cholesterol homeostasis remain scarce. The present study aims to investigate the precise role of IL-32 on HDLc homeostasis, focussing on cholesterol transporters involved in this process in human primary liver cells and the human monocytic cell line THP1.

2. Materials and methods

2.1. Culturing primary human liver cells, HepG2 and THP-1 cell lines and carotid artery plaque tissue

Patients undergoing liver surgery in our hospital donated human liver tissue. The anonymized liver tissue was used to isolate primary human liver cells as previously reported [32]. Human liver cells were seeded in Williams B medium containing 10% human serum and penicillin/streptomycin. This study (2014-1453) was reported and approved by our ethical committee of the Radboud University Medical Center. The human liver cell line HepG2 was cultured in complete Dulbecco's Modified Eagle's Medium with glutamax (Gibco) containing 10% heat-inactivated Fetal Calf Serum (FCS), pyruvate (Gibco) and gentamycin (Gibco). Trypsin was used to detach the adherent cells and the cell line was passaged twice a week in a 1:5 ratio.

The human monocytic cell line THP1 was used to study over-expression and silencing of IL-32 and its effect on cholesterol transporters. The cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing heat-inactivated 10% FCS, pyruvate and gentamycin. Cells were growing in suspension and passaged twice a week in fresh medium.

Fresh carotid artery plaque tissue was kindly provided by the department of surgery from the Radboud University Medical Center, and upon arrival was separated into 4 pieces to perform various analyses. Samples were then stored at -80°C for further use.

2.2. Quantitative PCR

Human primary liver cells were seeded ~200,000 cells per well in a 24 well plate in 0.5 ml Williams B medium containing 10% human serum and penicillin/streptomycin. The next day, cells were stimulated with TNF α (100 ng/ml) (R&D Systems) or Poly I:C (50 $\mu\text{g}/\text{ml}$) (Invivogen) for 24 h. Subsequently, medium was removed and stored and 0.5 ml Tri-reagent (Sigma-Aldrich) was added per well. After lysing the cells by incubating them with Tri-reagent for 30 min at room temperature, the solution containing lysed cells was stored at -20°C until further processing. HepG2 cells and THP-1 cells were lysed in TriZol reagent and also stored for later use. Carotid artery tissue was crushed in TriZol reagent using Magnalyser green beads (Roche). RNA was isolated as previously described [33]. After RNA isolation, mRNA was transformed into cDNA by applying an iScript kit (Bio-Rad) to transform mRNA into cDNA. IL-32 primers were previously developed and other primer sequences were extracted from the Harvard Primerbank database [34]. Primers were produced by Biolegio (Nijmegen, The Netherlands) and a StepOnePlus qPCR system (Applied Biosystems) was used to analyze relative mRNA expression. Relative expression was calculated by normalizing for GAPDH and $2^{-\Delta\text{Ct}}$ method.

2.3. Western blotting

Twenty-four hours after stimulation, primary liver cells were washed with PBS and lysed with standard cell lysis buffer on ice for 30 min. Subsequently, cells were scraped with a cell scraper and

transferred into a safe-lock tube. Proteins were boiled in Laemmli buffer under denaturing and reducing conditions. SDS-Page gels (12%) were prepared and marker plus proteins were loaded onto the gels. After running the gels, proteins were transferred onto nitrocellulose membranes with an iBlot apparatus (Invitrogen). After transferring the proteins, blots were blocked with 5% milk proteins in Tris-buffered Saline (TBS) with 0.1% tween-20 (Invitrogen) for at least 1 h at room temperature. Next, blots were washed in TBS-T (TBS containing 0.1% tween-20) and incubated overnight on the roller mixer at room temperature in TBS-T containing primary antibody against IL-32 (AF3040, R&D Systems), at a concentration of 0.2 µg/ml, or against ApoA1 (Acris Antibodies GmbH, Herford, Germany), at a concentration of 1.0 µg/ml. The following day, blots were washed with TBST and the IL-32 blots were incubated with rabbit-anti-mouse-HRP (Dako P0449, 1:5000 in TBST) while the ApoA1 blots were incubated with rabbit-anti-mouse-HRP (Dako P0260, 1:5000 in TBST) and incubated for 1 h at room temperature on the roller mixer. Next, blots were washed and incubated with ECL (GE Health care Life Sciences) before scanning the blots. Finally, actin was detected on the blots by using anti-actin antibodies (Santa Cruz Biotechnology) and appropriate secondary antibody (Dako) followed by ECL incubation and scanning of the blots.

2.4. IL-32 ELISA

Maxisorp plates (Nunc) were coated with AF3040 (R&D Systems) diluted in Phosphate Buffered Saline (PBS) at a concentration of 0.4 µg/ml and incubated overnight at room temperature. Next morning, plates were blocked with PBS containing 1% BSA (Sigma-Aldrich) for 1 h at room temperature. Standard curve was prepared by diluting recombinant IL-32 ranging from 5000 pg/ml until 39.06 pg/ml in PBS containing 5% BSA. Standard curve was added followed by the supernatant samples from the primary liver cells. ELISA plates were incubated for 2 h on a shaker. After the incubation, plates were washed and detection antibody was added (BAF3040, R&D Systems), 0.1 µg/ml in PBS with 5% BSA. Plates were incubated for 1 h at room temperature on the shaker. Subsequently, plates were washed and streptavidin (R&D Systems) was added and the plates were incubated for 30 min at room temperature on the shaker. After the last incubation, plates were washed and substrate buffer was added and the color reaction was closely monitored until the reaction was terminated by adding stop solution. Finally, the plates were read by a plate-reader and IL-32 concentrations were calculated.

2.5. Overexpression of IL-32 in HepG2 cell line

Half million HepG2 cells were seeded per well in a 24-well plate in RPMI-1640 including 5% FCS and incubated overnight at 37 °C and 5% CO₂. The following day, medium was replaced by the same fresh medium and transfection medium containing IL-32 plasmids (pCDNA3-IL32α, pCDNA3-IL32β, pCDNA3-IL32γ) or a control plasmid (pCDNA3-eGFP) was prepared. Transfection medium per transfection was prepared as follow; 1) dissolve 0.5 µg plasmid DNA in 25 µl serum free RPMI-1640, 2) add 1.5 µl Fugene HD (Promega) directly to the DNA mixture, 3) vortex 1–2 s and incubate for 15 min at room temperature, 4) add 25 µl dropwise per well while gently shaking the plate, 5) incubate for 24 or 48 h at 37 °C and 5% CO₂. After the incubation, RNA and protein samples were isolated for determining gene expression and IL-32 protein expression as previously described.

2.6. BODIPY flowcytometry assay

Transfected HepG2 cells and THP-1 cells were used for the BODIPY FACS analysis. In more detail, 1 million (HepG2) or 2.5×10^5 (THP-1) cells per well were used for the transfection after which cells were detached, spun down and resuspended in PBS containing 4% Formaldehyde (FA) for 15 min on ice. Thereafter, the cells were spun down again and resuspended in PBS with 5% BSA and BODIPY (1:500) for 45 min on ice in the dark, after which the BODIPY signal was measured.

2.7. Overexpression and silencing of IL-32 in THP1 cells

THP-1 cells (15×10^6 cells/15 mL) were differentiated into macrophages (75 cm² – tissue culture flask; Corning) in RPMI-1640 including 10% FCS, PMA (Sigma-Aldrich) at 10 ng/ml, β-mercaptoethanol (Sigma-Aldrich) at 50 µM and incubated for 48 h at 37 °C and 5% CO₂. 2.5×10^6 cells/800 µL were electroporated with Amaxa Nucleofactor technology (Lonza, Basel), according with the protocol described in [35]. For IL-32 knockdown, 1 µg of ON-TARGETplus SMARTpool siRNA per transfection was used or 1 µg of ON-TARGETplus SMARTpool control siRNA (Dharmacon Inc.), sequences are described in [36]. For IL-32 overexpression, 0.5 µg of pCDNA3 plasmid expressing human IL-32γ of eGFP was used as control. Transfected cells (3×10^5 /100 µL) were plated on flat-bottom 96-well plates (Costar) and 100 µL of transfection medium was added. After 4 and 24 h, the cell monolayers were collected by adding 200 µL of TRIzol and stored at –80 °C until mRNA extraction. Comparable studies were performed to determine transfection (eGFP) efficiency, which was around 30%.

2.8. Foam cell formation of THP-1 cells and human derived percoll monocytes

THP-1 cells (15×10^6 cells/15 mL) were differentiated into macrophages (75 cm² – tissue culture flask; Corning) in RPMI-1640, including 10% FCS, PMA (Sigma-Aldrich) at 10 ng/mL, β-mercaptoethanol (Sigma-Aldrich) at 50 µM, and incubated for 48 h at 37 °C and 5% CO₂. 5×10^5 cells per well were seeded in 24 wells plate and starved for 4 h in RPMI-1640 supplemented 2 mM L-glutamine, 1 mM pyruvate and 50 µg/ml gentamicin (GIBCO Invitrogen, Carlsbad, CA), after which oxidized LDL was added (25 µg/mL) for 24 h at 37 °C and 5% CO₂. Human peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll-Paque. Next, Percoll isolation of monocytes was performed as previously described [37]. In brief, $150\text{--}200 \times 10^6$ PBMCs were layered on top of a hyper-osmotic Percoll solution and centrifuged for 15 min at 580g. The interphase layer was isolated and cells were washed with cold PBS. Cells were resuspended in RPMI+++. An extra purification step was added by adhering Percoll isolated monocytes to polystyrene flat bottom plates (Corning, NY, USA) for 1 h at 37 °C and 5% CO₂; subsequently, a washing step with warm PBS was performed to yield maximal purity. Once Percoll monocytes were obtained, they were differentiated into macrophages for 6 days in 10% human pool serum. On day 6, medium was removed and cells were starved for 4 h, similar to the THP-1 cells, before being fed oxidized LDL for 24 h. After 24 h, supernatants were collected and cells were stored in TRIzol reagent for mRNA isolation.

2.9. Statistics

Statistical analysis were performed with the Mann-Whitney U test, Spearman correlation test or One-way Anova including Kruskal-Wallis test and Dunn's Multiple comparison test. In each figure, the applied statistical test is indicated.

3. Results

3.1. Induction of IL-32 isoforms in human primary liver cells by TNF α or TLR3 ligand poly I:C

Primary liver cells were stimulated with recombinant human TNF α (rhTNF α) or Poly I:C to study whether these cells were capable of expressing IL-32. Stimulation of cells with rhTNF α resulted in a slight upregulation of IL-32 α mRNA expression (Fig. 1A). Moreover, the protein level of IL-32 β was determined intracellularly and was induced mostly by rhTNF α , but also Poly I:C stimulation (Fig. 1D and E). Poly I:C stimulation resulted in a slight increase in IL-32 γ mRNA expression but almost no to minor increase in either IL-32 β or IL-32 α , respectively, compared to medium control (Fig. 1A–C). Furthermore, Poly I:C stimulation resulted in higher extracellular IL-32 protein levels (Fig. 1F).

3.2. Induction of IL-32 in human primary liver cells leads to enhanced expression of important regulators of cholesterol homeostasis which correlate strongly with IL-32 γ mRNA expression

Here, we explored whether TNF α - or TLR3-poly I:C-induced IL-32 expression in human primary liver cells modulates the expression of cholesterol transporters and regulators of cholesterol homeostasis. Furthermore, we studied whether there was a

correlation between mRNA expression of IL-32 isoforms and the components involved in the cholesterol homeostasis. As shown in Fig. 2A–D, stimulation of human primary liver cells with poly I:C resulted in increased expression of ABCA1, ABCG1, ApoA1 and LXR α mRNA. Besides mRNA levels, protein levels of ApoA1 were also studied and showed to be increased after stimulation with poly I:C, and possibly after rhTNF α stimulation (Fig. 2E and F). As a positive control to study the function of the cells, we determined the rhTNF α - and poly I:C-induced IL-8 mRNA expression in liver cells (Supplementary Fig. 1A). Additionally, a strong positive correlation between the IL-32 γ isoform and ABCA1, ABCG1, ApoA1 and LXR α expression was observed after the cells were stimulated with poly I:C (Fig. 2F–I). Cells stimulated with rhTNF α also showed positive correlations to the same mediators (Supplementary Figs. 1B–E). These correlations were not observed when looking at IL-32 α nor IL-32 β expression and these mediators (Supplementary Table 1).

3.3. Endogenous expression of IL-32 γ and ABCA1 mRNA in HepG2 cells

To further study the effect of IL-32 isoforms on the expression of cholesterol mediators, a human liver cell line was used (HepG2 cell line) (Fig. 3A–D). At first, unstimulated HepG2 cells were studied to look at the basal mRNA expression levels of IL-32 and cholesterol mediators. Similar to the observation made in human primary liver

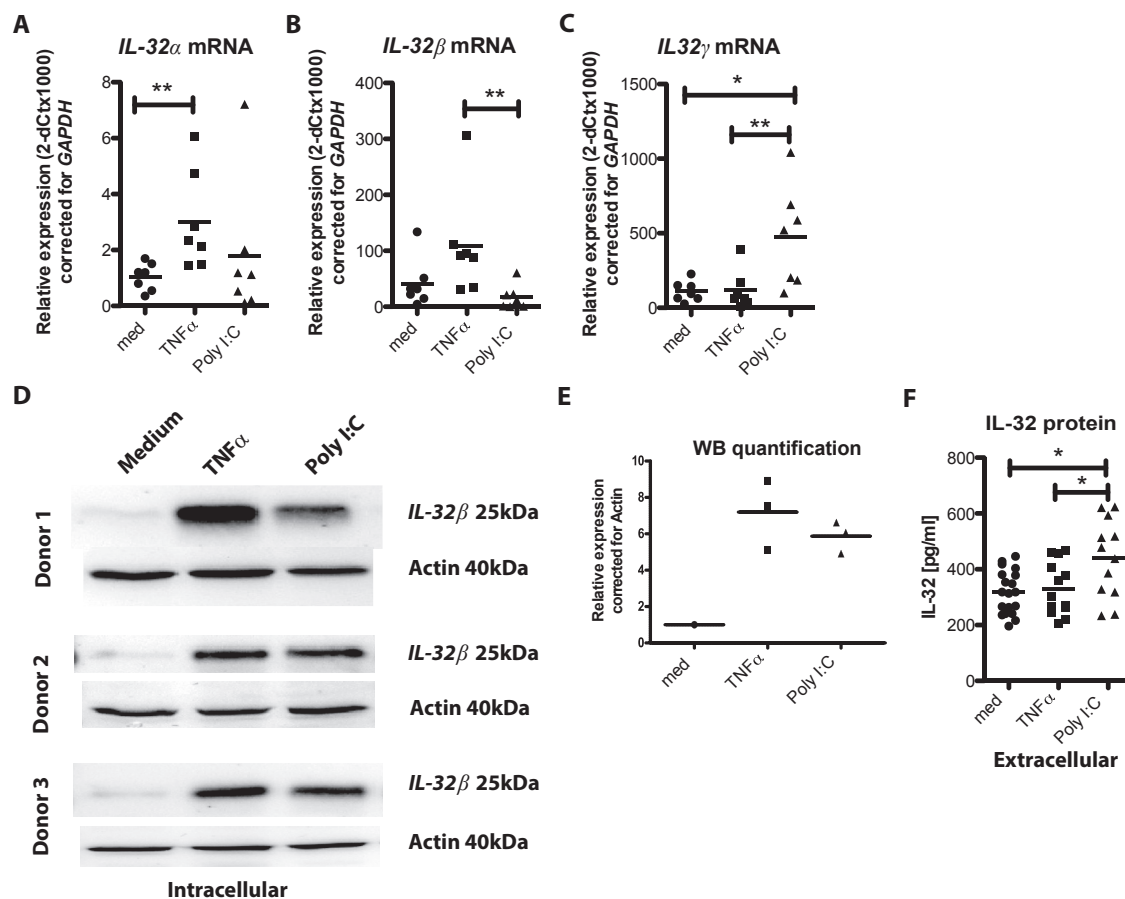


Fig. 1. Induction of IL-32 expression in human primary liver cells.

(A) Stimulation with TNF α induced significant expression of IL-32 α ($n = 7$, Mann-Whitney U test, $p = 0.0070$). (B) Significant differences between TNF α and Poly I:C induced IL-32 β ($n = 7$, Mann-Whitney U test, $p = 0.0023$). (C) Poly I:C stimulation enhanced the expression of IL-32 γ both compared with medium control ($n = 7$, Mann-Whitney U test, $p = 0.0111$) and TNF α ($n = 7$, Mann-Whitney U test, $p = 0.0070$). (D) Induction of IL-32 β protein by TNF α or Poly I:C in primary liver cells from 3 donors (actin as loading control). (E) Relative expression of IL-32 β protein corrected for actin expression after stimulation (med, TNF α , Poly I:C). (F) IL-32 protein expression after TNF α and Poly I:C stimulation in culture supernatants of human primary liver cells ($n = 5$ (with replicates), Mann-Whitney U test, medium vs. Poly I:C $p = 0.0176$; TNF α vs. Poly I:C $p = 0.0471$).

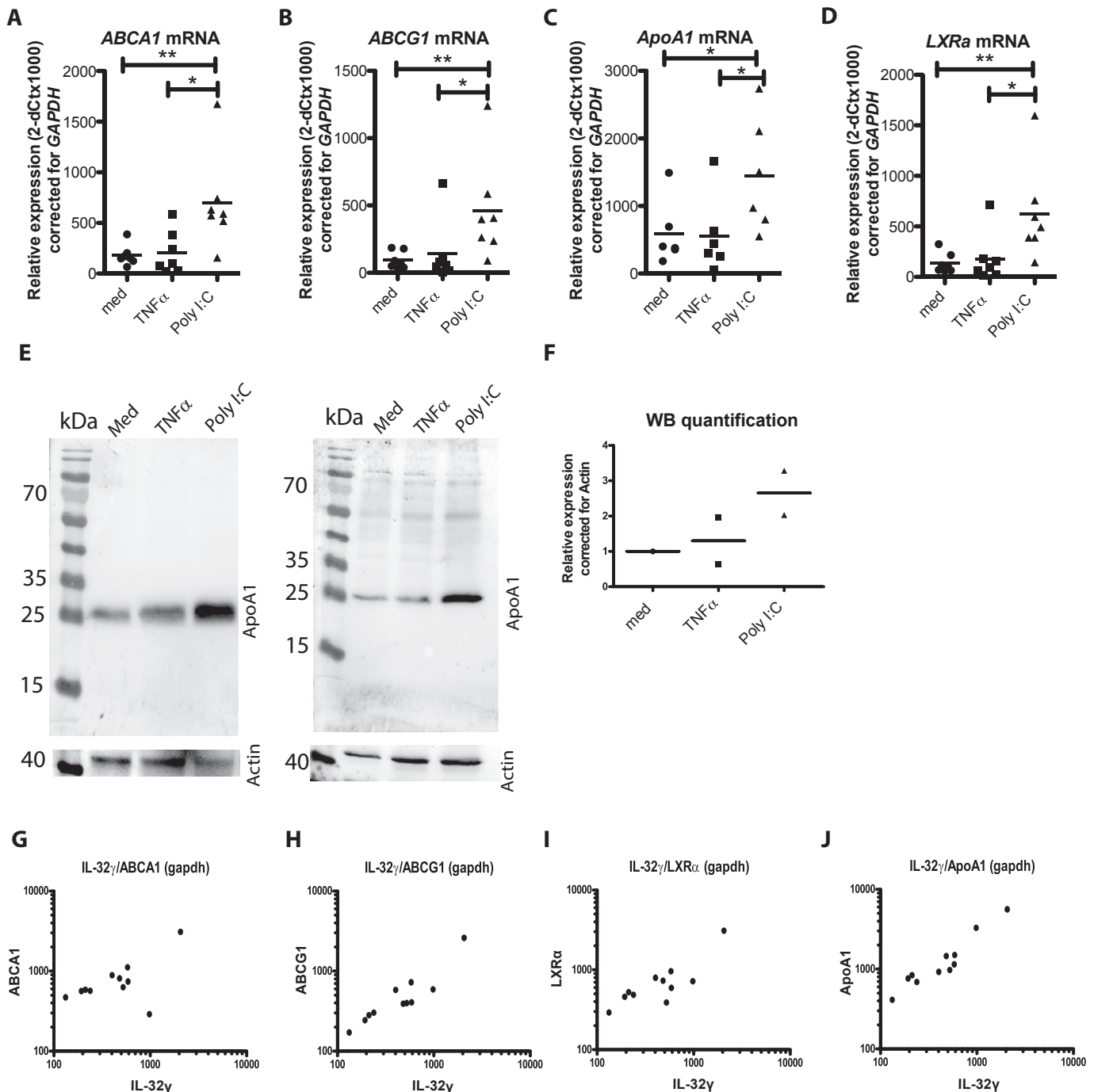


Fig. 2. Induction of cholesterol transporters ABCA1/ABCG1, HDL particle ApoA1 and transcription factor LXRα in human primary liver cells, and their correlation with IL-32γ after Poly I:C stimulation of cells for 24 h.

(A) Stimulation with Poly I:C significantly induced *ABCA1* ($n = 7$, Mann-Whitney U test, medium vs. Poly I:C $p = 0.0041$; TNFα vs. Poly I:C $p = 0.011$). (B) *ABCG1* expression was significantly induced by Poly I:C ($n = 7$, Mann-Whitney U test, medium vs. Poly I:C $p = 0.0023$; TNFα vs. Poly I:C $p = 0.0262$). (C) HDL particle *ApoA1* was significantly induced by Poly I:C ($n = 7$, Mann-Whitney U test, medium vs. Poly I:C $p = 0.0260$; TNFα vs. Poly I:C $p = 0.0411$). (D) Poly I:C stimulation significantly induced the transcription factor *LXRα* ($n = 7$, Mann-Whitney U test, medium vs. Poly I:C $p = 0.0023$; TNFα vs. Poly I:C $p = 0.0262$). (E) Induction of ApoA1 protein after Poly I:C stimulation in 2 different donors. (F) Relative expression of ApoA1 protein corrected for actin expression in two donors. (G) Positive correlation between IL-32γ and *ABCA1* (Spearman $r = 0.4727$, $p = ns$). (H) Positive correlation between IL-32γ and *ABCG1* (Spearman $r = 0.9091$, $p = 0.0003^{***}$). (I) Positive correlation between IL-32γ and *LXRα* (Spearman $r = 0.6726$, $p = 0.0277^*$). (J) Positive correlation between IL-32γ and *ApoA1* (Spearman $r = 0.9455$, $p < 0.0001^{***}$). All graphs show the relative mRNA expression adjusted for *GAPDH* expression. ($n = 5$, data includes replicates).

cells, also HepG2 cells showed expression of the three isoforms of IL-32 (*IL-32α*, *IL-32β* and *IL-32γ*) and *ABCA1* (Fig. 3A–D). Interestingly, IL-32γ expression was already upregulated after 4 h of culture in only DMEM complete medium with 10% FCS hi, together with *ABCA1* expression, while IL-32α did not show any expression and IL-

32β only minor expression (Fig. 3A and B). As shown in Fig. 4A and B, the relative expression of *IL-32α* and *IL-32β* increased after 24 h, but resulted in an even more pronounced expression after 48 h of culture. Within the same time, expression of both *IL-32γ* and *ABCA1* decreased (Fig. 3C and D). Highly interesting, a positive correlation

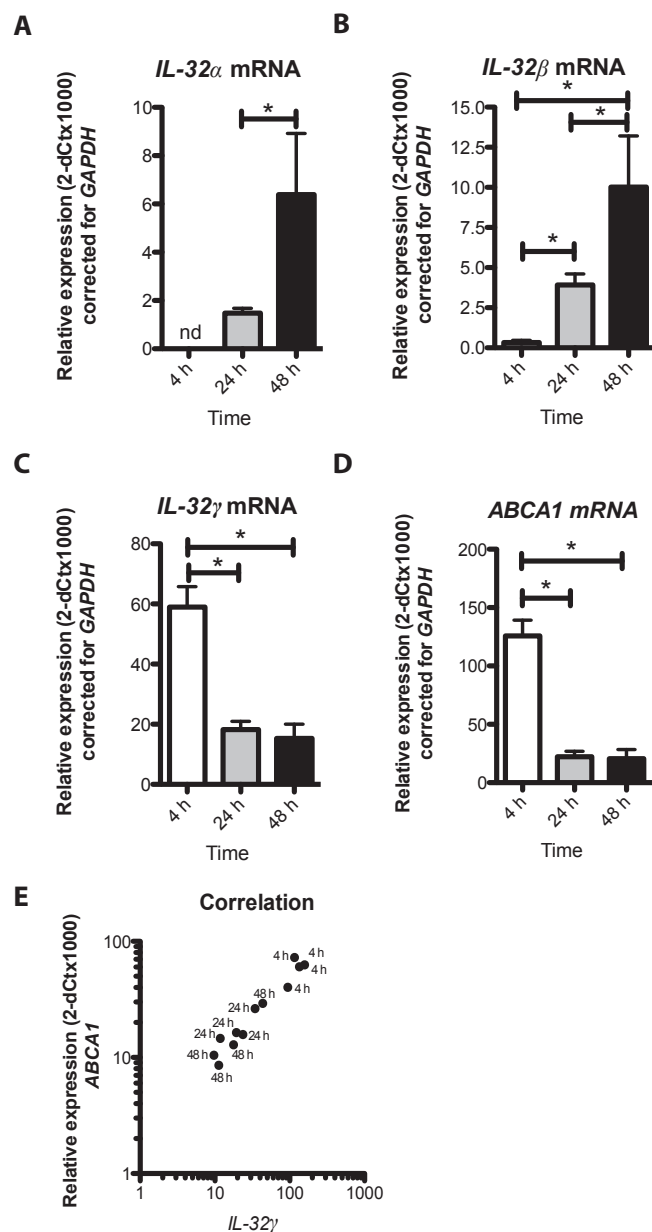


Fig. 3. Time-dependent induction of IL-32 γ and cholesterol transporter ABCA1 in human HepG2 cells.

(A) Expression of IL-32 α is significantly induced after 48 h compared with 24 h, while at 4 h, the expression of IL-32 α was not detectable (4 replicates, Mann-Whitney U test, $p = 0.0286$). (B) Expression of IL-32 β was already detected at 4 h and significantly enhanced after 24 h and after 48 h (4 replicates, Mann-Whitney U test, $p = 0.0286$). (C) Expression of IL-32 γ was already high at 4 h and decreased after 24 h and 48 h (4 replicates, Mann-Whitney U test, $p = 0.0286$). (D) Expression of the cholesterol transporter ABCA1 was high at 4 h and significantly decreased after 24 h and 48 h (4 replicates, Mann-Whitney U test, $p = 0.0286$). (E) Correlation between IL-32 γ and ABCA1 at different time points showed to be highly significant $p < 0.0001$, with a Spearman r of 0.9580. All conditions were kept in DMEM complete medium with 10% fetal calf serum heat-inactivated. Time course started after plating the cells.

between IL-32 isoforms and ABCA1 expression was again mostly observed for the IL-32 γ isoform (Fig. 3E and Supplementary Fig. 2).

3.4. Overexpression of IL-32 α , IL-32 β or IL-32 γ results in strong induction of cholesterol transporters and reduced intracellular lipid concentrations in HepG2 cells

Since we noted strong correlations between mRNA expression

of IL-32 isoforms and cholesterol transporters, we were interested in the function of these cholesterol transporters in the presence of IL-32. HepG2 cells were therefore transfected with pCDNA3 constructs containing either eGFP (as a negative control), IL-32 α , IL-32 β or IL-32 γ . As shown in Fig. 4A, overexpression of the various isoforms of IL-32 resulted in protein expression of that specific isoform after 48 h of transfection. Moreover, overexpression of the IL-32 α isoform resulted in a trend towards an increased expression in LXR α and ABCA1 (Fig. 4B and C). Additionally, overexpression of the IL-32 β and IL-32 γ isoforms resulted in an increased expression of LXR α (non-significant for IL-32 γ), ABCA1 and ABCG1 (Fig. 4B–D). However, important mediators such as ApoA1 (mRNA and protein) and SR-B1 (mRNA) were not affected in HepG2 cells overexpressing the isoforms of IL-32 (Supplemental Fig. 3). Finally, we performed a boron-dipyrromethene (BODIPY) staining to study the intracellular lipid content of the transfected versus untransfected HepG2 cells. BODIPY fluorescence was significantly lower in HepG2 cells transfected with the IL-32 β isoforms and showed a trend towards lower expression in IL-32 α and IL-32 γ transfected compared to the untransfected control or eGFP transfected negative control (Fig. 4E). The strongest reduction of BODIPY was observed after transfection of HepG2 cells with IL-32 β .

3.5. Silencing of IL-32 γ strongly reduces ABCA1 mRNA expression

As described above, overexpression of the IL-32 γ isoform in HepG2 liver cells resulted in an increased expression and possible function of ABCA1. To study the role of IL-32 γ into more detail, we studied IL-32 γ and ABCA1 expression in carotid artery plaque tissue and performed knockdown experiments of IL-32 γ . Due to the fact that HepG2 cells did not survive the procedure to knockdown IL-32 γ , we were prompted to use another cell line. We performed silencing and overexpression experiments in a human monocytic cancer cell line (THP1 cell line). This because ABCA1 expression and function is also very important for monocytes in the circulation, contributing to reverse cholesterol transport. Using THP1 cells, IL-32 γ overexpression resulted in high expression of IL-32 γ as well as ABCA1 (Fig. 5A and D). When IL-32 γ was silenced, IL-32 γ and ABCA1 expression was completely diminished (Fig. 5B and E). This effect was not caused by the transfection method itself, since THP1 cells transfected with eGFP or spC negative controls did not result in these effects on ABCA1 expression (Fig. 5A, B, D and E). The spIL-32 construct is also capable of silencing other isoforms of IL-32 as is shown by the silencing of IL-32 β (Fig. 5C). Besides, since we observed similar expression patterns of induced cholesterol transporters in THP-1 cells, we performed overexpression experiments in THP-1 cells to do the BODIPY staining. Overexpressing IL-32 γ in THP-1 cells resulted in a decrease of BODIPY fluorescence (Supplementary Fig. 4). Moreover, after observing the importance of IL-32 γ on ABCA1 expression in liver cells and THP-1 cells, we were curious if this effect was also present in plaque tissue in which ABCA1 expression can affect foam cell formation. We were able to show an increased expression of IL-32 γ and ABCA1 mRNA in carotid artery plaque tissue (Fig. 5F and G). Finally, THP-1 derived macrophages and human derived percoll monocytes (differentiated to macrophages) were loaded with oxidized LDL (25 μ g/mL, 50 μ g/mL, respectively) for 24 h to study the expression of IL-32 isoforms and mediators involved in cholesterol metabolism in foam cells. Interestingly, IL-32 isoform expression of IL-32 α , IL-32 β and IL-32 γ seemed to be decreased or unchanged compared to normal macrophages (Supplementary Fig. 5). Nevertheless, cholesterol mediators ABCA1, ABCG1 and LXR α seemed to be induced, albeit not significantly (Supplementary Fig. 5).

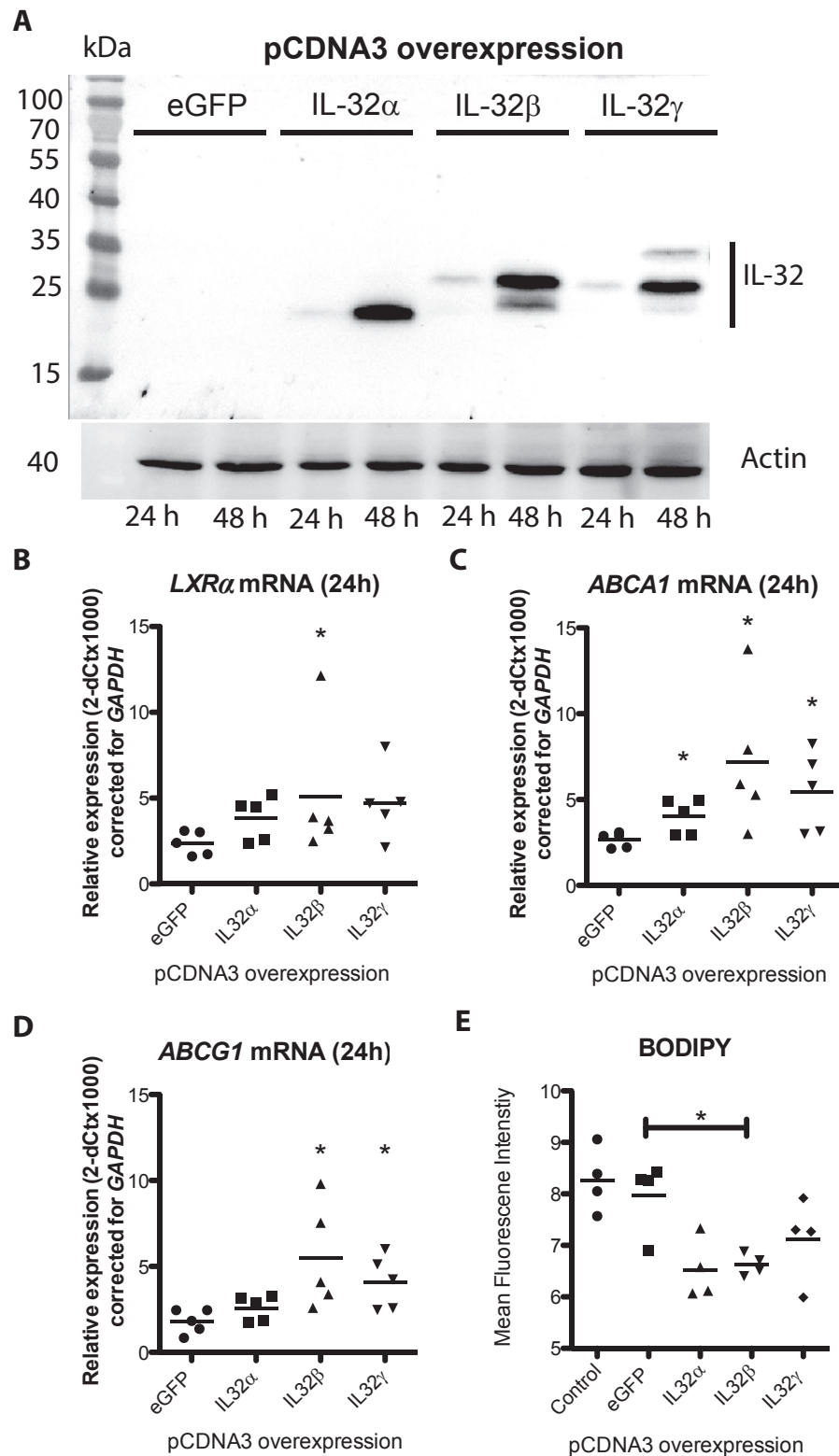


Fig. 4. Modulation of transcription factor LXR α and cholesterol transporters ABCA1/ABCG1 resulted in lower intracellular lipid content after IL-32 overexpression in human HepG2 cells.

(A) Overexpression of different IL-32 isoforms in human HepG2 liver cells at 24 h and 48 h. IL-32 α (approx. 19 kDa), IL-32 β (approx. 26 kDa), IL-32 γ (approx. 29 kDa). (B) Transcription factor LXR α was significantly induced after overexpression of IL-32 β (5 replicates, Mann-Whitney *U* test, $p = 0.0317$). (C) Overexpression of IL-32 α ($p = 0.0317$), IL-32 β ($p = 0.0159$), IL-32 γ ($p = 0.0159$) significantly induced ABCA1 expression (5 replicates, Mann-Whitney *U* test). (D) Overexpression of IL-32 β and IL-32 γ enhanced the expression of ABCG1 (5 replicates, Mann-Whitney *U* test, $p = 0.0079$). (E) Mean fluorescent intensity was significantly reduced after overexpression of IL-32 β (4 replicates, Mann-Whitney *U* test, $p = 0.0286$). Replicates are from 2 independent experiments with technical replicates within each experiment.

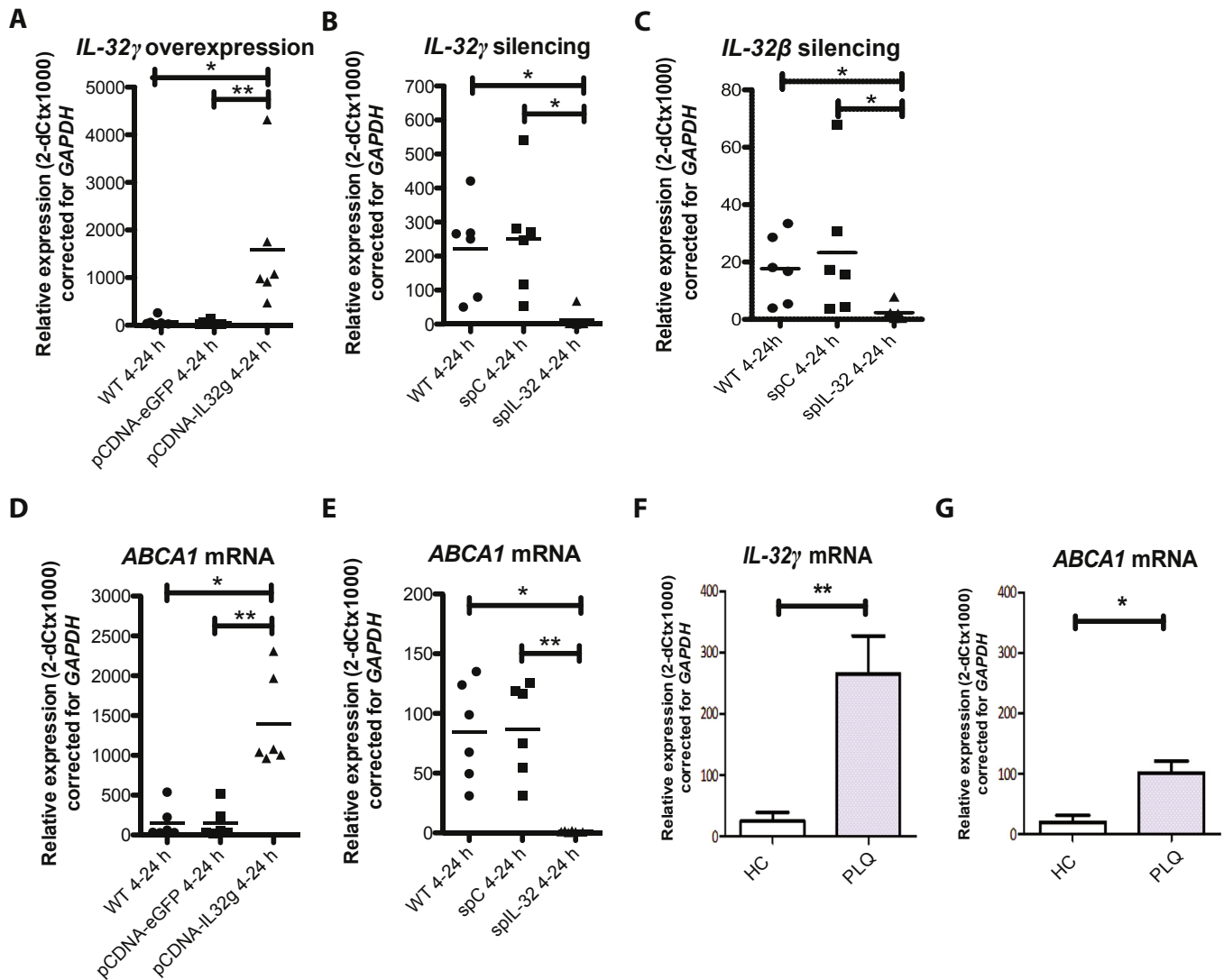


Fig. 5. IL-32 regulates cholesterol transporter ABCA1 in THP1 cells and is expressed in human carotid artery plaque tissue.

(A) Human THP1 cells transfected with different plasmids (pCDNA3-eGFP or pCDNA3-IL32 γ) or no plasmids (wt control) showed significant induction of IL-32 γ when the cells were transfected with pCDNA3-IL32 γ ($p < 0.05^*$ or $p < 0.01^{**}$). 6 replicates, One-Way ANOVA, Kruskal test, Dunn's Multiple Comparison Test. (B) Silencing of IL-32 γ showed significant reduction of IL-32 γ expression compared to the wt control or smartpool-control (6 replicates, One-Way ANOVA, Kruskal-Wallis test, Dunn's Multiple Correction Test). (C) Silencing of IL-32 β showed significant reduction of IL-32 β expression compared to the wt control or smartpool-control (6 replicates, One-Way ANOVA, Kruskal-Wallis test, Dunn's Multiple Correction Test). (D) Overexpression of IL-32 γ induced significant expression of ABCA1. (E) Silencing of IL-32 γ downregulated ABCA1 significantly in THP1 cells. (F) Human carotid artery plaque tissue showed induced expression of IL-32 γ mRNA compared to healthy tissue ($p = 0.0017^*$). (G) Human carotid artery plaque tissue showed induced expression of ABCA1 mRNA compared to healthy tissue ($p = 0.0124^*$).

4. Discussion

In the present study, we aimed to investigate the effect of IL32 on proteins involved in cholesterol metabolism in liver cells and atherosclerotic plaques. We show for the first time that both human primary liver cells and a HepG2 cell line express IL-32 mRNA and protein and that human carotid artery tissue expresses IL-32 γ mRNA. Moreover, the three main IL-32 isoforms, IL-32 γ , IL-32 β and IL-32 α , can be induced either by recombinant human (rh)TNF α or a synthetic analogue of a double-stranded RNA virus (Poly I:C) stimulation in these liver cells. mRNA expression of components important in cholesterol metabolism, such as, ABCA1, ABCG1, LXR α and apoA1, are correlated to expression levels of IL-32 γ isoforms in unstimulated human primary liver cells and HepG2 cells. Additionally, overexpression of IL-32 β and IL-32 γ resulted in induction of ABCA1 and ABCG1 in HepG2 cells and overexpression of IL-32 γ

induction of ABCA1 expression in THP-1 cells. By performing the BODIPY flowcytometry assay, we could show that in the presence of endogenous IL-32, intracellular lipid concentrations were decreased. Moreover, silencing of IL-32 in THP-1 cells caused a strong reduction of ABCA1 expression. Lastly, we also observed increased mRNA expression of IL-32 and ABCA1 in human carotid artery tissue obtained from carotid endarterectomy surgery.

A recent study suggested the existence of an association between IL-32 and HDL cholesterol, which can become relevant for patients with an increased risk to develop CVD, such as RA patients. A single nucleotide polymorphism (SNP) in the promoter region of IL32 was described to be correlated with higher HDLc concentrations in RA patients, suggesting a possible role for IL-32 in determining CVD risk [18]. Moreover, this SNP possibly results in more IL-32 protein expression [38]. This suggests higher IL-32 protein concentrations are linked with higher HDLc concentrations.

Next to IL-32 mRNA and protein, we also studied the expression of various cholesterol transporters/mediators involved in cholesterol metabolism such as, ABCA1, ABCG1, ApoA1 and LXR α . Tight regulation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol is important to prevent cardiovascular disease, such as atherosclerosis. Circulating HDLc concentrations are regulated by biosynthesis and degradation processes in which the liver plays an important role [22]. HDL biosynthesis is regulated by the production and secretion of apoA-I by the liver and lipidation of these apolipoproteins by ABCA1 transporters expressed on liver cells [23]. Expression of this cholesterol transporter ABCA1 in hepatocytes plays an important role in the biosynthesis and regulation of circulating HDL cholesterol [26,39,40]. Furthermore, enterocytes are also involved in the biosynthesis and maintenance of HDL. Via a complex network of cholesterol transporters, the small intestine establishes a balance between the amount of excreted and absorbed cholesterol and in that way influences RCT [41,42]. Moreover, downregulating absorption of cholesterol in the intestine has been shown to improve RCT [41]. Besides, degradation of HDLc is regulated by different pathways. One way the body can get rid of circulating HDLc is by uptake of HDLc via the SR-B1 transporter on liver cells. An alternative pathway is the degradation of HDLc by CETP. A third way HDLc can be affected is by catabolism in the kidney via cubilin endocytosis and SRB1 in the proximal tubulus of the kidney. HDLc will be reabsorbed and degraded mostly via SRB1 whereas cubulin and megalin endocytose essentially lipid-free apoA-I [43,44]. These changes in HDL composition occur during the important RCT pathway. Excessive cholesterol is transported from the peripheral tissues, such as the vessel wall, back to the liver for excretion, preventing atherosclerosis. During RCT, accumulated cholesterol from macrophages in the vessel wall is removed to HDL or lipid-poor apolipoprotein (apo)A1 by different mechanisms, including one which is dependent on the cell membrane expression of ABCA1 on macrophages [45,46]. Upregulation of ABCA1 would therefore favour an anti-atherogenic environment whereas downregulation of ABCA1 could create an atherogenic state by reducing cholesterol efflux and HDLc concentrations. The aim of the study was to understand the mechanism behind the regulation of HDLc concentrations by IL-32. Our results show that induction of IL-32 γ is correlated with induced ABCA1 mRNA expression in unstimulated human primary liver cells and HepG2 cells. IL-32 β expression did not show any correlation and IL-32 α even showed a negative correlation with ABCA1, ABCG1 and LXR α mRNA expression. Additionally, overexpression experiments of IL-32 isoforms in HepG2 cells showed that IL-32 β and IL-32 γ are associated with upregulation of ABCA1 and ABCG1. Due to the fact that HepG2 cells were no longer viable after knocking down IL32, overexpressing and silencing experiments of IL-32 γ were performed in THP1 macrophages. In the presence or absence of IL-32 γ , ABCA1 expression was strongly induced or reduced, respectively. This definitely demonstrated that IL-32 γ is a key player in driving ABCA1 expression. We suggest that this effect is most likely caused by the direct regulation of LXR α expression by IL-32. LXR α is known to mediate, at least partially, the expression of ABCA1 [47]. Furthermore, we show that the most potent isoform of IL-32, IL-32 γ , together with ABCA1, ABCG1 and LXR α , is also strongly upregulated by Poly I:C stimulation of primary liver cells and HepG2 cells. These data are conflicting with previous data by Castrillo A et al., who showed that LXR α and ABCA1 are inhibited after activation of Toll-like receptors (TLRs) 3 and 4 by microbial ligands like viruses or bacteria [48]. One explanation for this could be the fact that two completely different cell types are used in our experiments compared to the macrophages used in Castrillo's experiments. Moreover, it is possible that stimulation of hepatocytes with a viral component such as poly I:C

could trigger a slightly different intracellular pathway or the expression of IL-32 in these cells is of more importance and therefore results in different effects in various cell types.

Besides, the changes we found on cholesterol transporters expression seem functional, since intracellular lipid concentrations were lower in cells overexpressing IL-32 isoforms. These data show a completely new function of endogenous IL-32 in liver cells and THP-1 derived macrophages even suggesting a possible anti-atherosclerotic function for the pro-inflammatory cytokine. However, when studying THP-1 macrophage foam cells or foam cells generated from human percoll monocytes differentiated to macrophages, isoforms of IL-32 were reduced or unchanged and only ABCA1, ABCG1 and LXR α were induced. This might be explained by the fact that oxidized LDL is taken up by different receptors such as CD36 and scavenger receptor A which could result in a different intracellular signal cascade compared to poly I:C stimulation or overexpressing experiments [49]. Another explanation for the new link between IL-32 and ABCA1 could be explained by the fact that ABCA1 also has other important functions besides regulating HDL cholesterol. Previous studies showed that intracellular cholesterol homeostasis was required for a housekeeping function of cells. Additionally, recent studies indicated that cholesterol regulation, more specifically sterols, are dynamically regulated, bioactive and are intrinsic players in the immune response that couples metabolism to host defence [50]. The question whether IL-32 is anti- or pro-atherogenic is still difficult to answer. For now, we can conclude that IL-32 has many pro-inflammatory/pro-atherogenic capacities but that this study shows that IL-32 γ closely associates with ABCA1 and other cholesterol mediators and in that way serves as an anti-atherogenic mediator.

Some limitations could be envisaged in our study. One of these limitations could be the fact we did not measure HDL functionality but only HDLc concentrations even though previous studies have showed functionality being more important than HDL cholesterol concentration in determining cardiovascular disease risk [19,51]. Furthermore, additional experiments could not be performed due to lack of sample since all samples were used for the initial experiments.

To conclude, the present study shows for the first time the existence of a direct association between IL-32 and cholesterol homeostasis in humans. We provide clear and novel evidence that IL-32 is an important regulator of cholesterol transporters ABCA1 and ABCG1, possibly explaining the variation of HDLc concentrations previously observed in individuals bearing a SNP in IL-32 gene. Whether these interactions would further translate into a higher or lower CV risk remains to be elucidated in future studies, as well as the gain of more knowledge and understanding of the complex interactions between inflammatory effectors, lipids homeostasis and atherosclerosis.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Authors MSMAD, BH, LABJ and JCS RH, JAV, MGN, NPR and CAD contributed to the design of the study, acquisition of data, analysis

and interpretation of data. All authors furthermore contributed to drafting and critically revising the manuscript to create an approved version for submission to the journal.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.atherosclerosis.2018.02.027>.

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